NASA TECHNICAL TRANSLATION NASA TT F-15,731

# PROTEOLYTIC ENZYMES AND PROTEIN METABOLISM IN RABBIT ERYTHROCYTES DURING CELL MATURATION IN VIVO

R. J. Haschen, W. Farr and F. Groh

Translation of "Proteolytische Enzyme und Eiweissstoffwechsel der Kaninchen-Erythrozyten Waehrend der Zellreifung in Vivo," Acta Biologica et Medica Germanica, Volume 14, 1965, pages 205-215.

(NASA-TT-F-15731) PROTEOLYTIC ENZYMES AND PROTEIN METABOLISM IN RABBIT ERYTHROCYTES DURING CELL MATURATION IN VIVO (Techtran Corp.) 14 p HC $4.00 CSCL 06D

N74-27555

Unclas
G3/04 43156

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NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
WASHINGTON, D. C. 20546 JUNE 1974

STANDARD TITLE PAGE

| 1. Report No. | 2. Government Accession No. | 3. Recipient's Catalog No. |
|---|---|---|
| NASA TT F-15,731 | | |

| 4. Title and Subtitle | 5. Report Date |
|---|---|
| PROTEOLYTIC ENZYMES AND PROTEIN METABOLISM IN RABBIT ERYTHROCYTES DURING CELL MATURATION IN VIVO | JUNE 1974 |
| | 6. Performing Organization Code |

| 7. Author(s) | 8. Performing Organization Report No. |
|---|---|
| R. J. Haschen, W. Farr and F. Groh | 10. Work Unit No. |

| 9. Performing Organization Name and Address | 11. Contract or Grant No. |
|---|---|
| Techtran Corporation<br>P.O. Box 729, Glen Burnie, Md. 21061 | NASw-2485 |
| | 13. Type of Report and Period Covered |
| | Technical Translation |
| 12. Sponsoring Agency Name and Address | 14. Sponsoring Agency Code |
| National Aeronautics and Space Administration<br>Washington, D. C. 20546 | |

15. Supplementary Notes

Translation of "Proteolytische Enzyme und Eiweissstoffwechsel der Kaninchen-Erythrozyten Waehrend der Zellreifung in Vivo," Acta Biologica et Medica Germanica, Volume 14, 1965, pages 205-215.

16. Abstract

In the haemolysate of erythrocytes (reticulocytes) of rabbits treated and not treated with phenylhydrazine the authors determined the "acid proteinase" (pH 3.5), cathepsin A, 6 exopeptidases and aspartate-amino--transferase, and at the same time the total N, stroma-N, Hb-N, NPN and amino-N. The enzymes behave differently during the process of maturation: cathepsine A rapidly disappears; the "acid proteinase", glycyl--glycine-, glycyl-leucine- and imino-dipeptidase, as well the aspartate--aminotransferase show a marked decrease, while leucine-aminopeptidase is slightly decreased; the activities of aminotripeptidase and imino--dipeptidase remain unchanged. The time sequence of these changes was specified more precisely by serial determinations during cellular regeneration and maturation. These studies demonstrate illuminating correlations with regard to the metabolism of cellular proteins.

| 17. Key Words (Selected by Author(s)) | 18. Distribution Statement |
|---|---|
| | |

| 19. Security Classif. (of this report) | 20. Security Classif. (of this page) | 21. No. of Pages | 22. Price |
|---|---|---|---|
| Unclassified | Unclassified | ~~13~~ 14 | 4.00 |



NASA-HQ

# PROTEOLYTIC ENZYMES AND PROTEIN METABOLISM IN RABBIT ERYTHROCYTES DURING CELL MATURATION IN VIVO

R. J. Haschen, W. Farr and F. Groh[1]

Studies with clinical material [1, 2] have shown an increase in activity /205
of certain erythrocyte peptidases and aspartate amino-transferase with increased effective erythropoesis (hemolytic states, hemorrhagic anemia, reticulocyte crisis with treated sideropenic and pernicious anemia). The degree of the increase varied quantitatively, however. Thus, the activities of leucine-amino-peptidase, glycyl-leucine dipeptidase and aspartate amino-transferase increased to four times normal on the average. Tripeptidase on the other hand was only slightly increased or not at all.

It was therefore likely that the proteolytic enzymes decreased at different /206
rates during the maturation of the erythrocytes. The findings of Ellis et al. already point in this direction. In other enzyme systems, similar selectivity of the maturation changes has already been observed [4].

To clarify this problem, we determined as comprehensive as possible a range of proteolytic enzymes. At the same time, the behavior of various nitrogen fractions of the erythrocytes was studied. The experiments were divided into two parts. Initially the applicable parameters were determined in healthy and phenylhydrazine treated rabbits and the differences checked for significance. Then the nature of the changes with time was studied. This provided information about the interplay of the proteolytic enzymes and their functions in protein metabolism during cell maturation.

## Material and Methods

Rabbits weighing 2.1-3.6 kg were used. Following determination of the normal values, they each received 5.7 mg of phenylhydrazine in 47% alcohol/kg

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*Numbers in the margin indicate foreign pagination.

subcutaneously on 3 successive days and twice the dose on the fourth day. Blood was collected on the sixth day to produce a cell suspension rich in reticulocytes.

The isolation of the red blood corpuscles (at approximately 2°C), the production of the hemolysate and the method of enzyme determination have been described in a previous paper [5]. The reticulocytes were rendered visible by brilliant cresyl blue stain, fixing in methanol and contrast staining with Giemsa's solution.

Enzyme Determination. They were performed under practically the same conditions which have proven optimal for human hemolysate [5]. Since the leucine amino-peptidase of the rabbit erythrocytes shows a much higher activity than in human erythrocytes, the preparations in question contained only a hemolysate corresponding to 0.96 volume percent of packed erythrocytes. The preparations for determination of Cathepsin A [6] contained 10 mM Benzyloxy-carbonyl-L-Glutamyl-L-Tyrosine, 0.025 M $KH_2PO_4$/citric acid buffer (final pH = = 5.2) and a hemolysate corresponding to 4.8 volume percent of packed erythrocytes. Aerobic incubation for 6 hours at 37°C. Determination of the split-off Tyrosine was carried out using quantitative paper chromatography or by the ninhydrin method [7]. No activation by 10 mM L-Cysteine could be detected. The hydrolysis of Benzyloxycarbonyl-Glycyl-L-Phenylalanine was similar, but was 2.67 times slower. The optimum pH for the acid proteinase was 3.5.

Determination of Nitrogen Fractions.

Amino-N: Deproteinization of cell suspension with 3 volumes of 10% trichloroacetic acid, copper complex method [46].

Total-N: Kjeldahl's semimicro method; incineration of a 0.2 ml cell suspension, capture of the liberated $NH_3$ in 4% boric acid and titration with 0.1 N HCl.

Residual-N: Deproteinization of 0.2 ml with 2 ml $H_2O$ hemolyzed cell suspension with 2 ml of 20% trichloroacetic acid, centrifuging (1,400 g), N-determination in the supernatent fluid as well as total-N, titration with 0.01 N HCl.

Stroma-N: Isolation of the stromata by centrifuging at 3,800 g (2°C) and repeated washing with a 0.9% NaCl solution, N-determination as well as total-N, titration with 0.1 N HCl.

The Hb-N was obtained by dividing the mean corpuscular hemoglobin concentration (MCC) by 0.6.

The difference between the total N - (Hb-N + stroma-N + residual-N) was referred to as the protein residue. The ribonucleic acid-N contained herein was disregarded, since this fraction increases as the reticulocyte number decreases. /207

## Results

The results of the determinations before and after Phenylhydrazine treatment are shown in Table 1. A comparison of the enzyme activities of the untreated groups with those of healthy human beings [5] show a considerable agreement. A clear exception is that of leucine-amino-peptidase, which has an activity in rabbits which is six times that in man. In blood which is rich in reticulocytes most of the enzymes are more or less markedly increased. The average values of a treated and untreated groups were compared by means of the t-test.

TABLE 1. ERYTHROCYTOMETRIC FINDINGS, PROTEOLYTIC ENZYMES AND N-FRACTIONS IN RED BLOOD CELLS OF THE RABBIT, BEFORE (8 ANIMALS) AND AFTER (6 ANIMALS) TREATMENT WITH PHENYLHYDRAZINE

| | Untreated | Treated with Phenylhydrazine | P |
|---|---|---|---|
| Erythrocytometry | | | |
| Hemoglobin (g/100 ml of blood) | 8,74 ± 1,08[1] | 3,74 ± 1,28 | 0,001 |
| Reticulocytes (%) | 2,5 ± 0,47 | 61,5 ± 3,89 | 0,001 |
| MCV | 74,8 ± 6,1 | 134,0 ± 24,5 | 0,001 |
| MCH | 20,4 ± 1,45 | 28,0 ± 4,64 | 0,01 |
| MCC | 27,9 ± 1,44 | 21,4 ± 4,07 | 0,01 |
| Enzyme | Micromoles/hr/ml of cells (37°C) | | |
| "Acid Proteinase" | 3,2 ± 1,06 | 13,6 ± 3,69 | 0,001 |
| Cathepsin A | 0,0 | 12,5 ± 11,40[2] | — |
| Leucine-amino-peptidase (3.4.1.1) | 172 ± 10,9 | 208 ± 47,2 | 0,05 |
| Amino-tripeptidase (3.4.1.3) | 844 ± 104,5 | 909 ± 281 | n.s.[3] |
| Glycylglycin-dipeptidase (3.4.3.1) | 360 ± 41,8 | 1280 ± 150 | 0,001 |
| Glycyl-leucine-dipeptidase (3.4.3.2) | 1336 ± 622 | 8528 ± 3432 | 0,001 |
| Imidodipeptidase (3.4.3.7) | 299 ± 43,8 | 270 ± 17,3 | n.s. |
| Iminodipeptidase (3.4.3.6) | 395 ± 93,4 | 1073 ± 222 | 0,001 |
| Aspartate-amino-transferase (2.6.1.1) | 64,0 ± 21,7 | 132,2 ± 19,5 | 0,001 |

TABLE 1 (CONTINUED)

| N-fractions | Untreated | Treated with Phenylhydrazine | P |
|---|---|---|---|
| | mg N/ml cells | | |
| Residual-N | 0,547 ±0,142 | 1,145 ±0,393 | 0,01 |
| Amino-N | 0,157 ±0,018 | 0,292 ±0,127 | 0,01 |
| Hb-N | 46,5 ±2,40 | 35,7 ±6,79 | 0,01 |
| Stroma-N | 1,32 ±0,465 | 4,09 ±1,093 | 0,001 |
| Protein residue | 8,95 ±3,27 | 13,39 ±4,79 | n.s. |
| Total-N | 57,27 ±5,955 | 54,33 ±3,800 | n.s. |

Commas indicate decimals.

[1]Arithmetic mean and standard deviation of the individual determinations.
[2]Boundary values: 2.67 and 27.8 micromoles/hour/ml of cells.
[3]Not significant.

In the last of Table 1, the error probability (P) is given. According to /208
this value, amino-tripeptidase and imido-dipeptidase have equal activities in erythrocytes and reticulocytes. Leucine-amino-peptidase is slightly increased in the reticulocytes; the difference with respect to the erythrocytes is only slightly ensured. In orientational experiments, L-Leucyl-β-Naphthylamide was also used as substrate (1.37 mM, pH 6.8, determination of the liberated leucine by quantitative paper chromatography). In the case of erythrocytes and reticulocytes (64%) the activity was similarly 16.8 micromoles/hour/ml of cells. It is still a moot point whether this activity can be attributed to the leucine--amino-peptidase alone or whether there is some other amino-peptidase which is responsible (see [8, 9, 47]). The other enzymes exhibit a clearly increased activity in the reticulocytes. The quotients of the activities of the reticulocytes and erythrocytes for acid proteinase are 4.3, for glycyl-glycine--dipeptidase 3.6, glycyl-leucine-dipeptidase 6.4, imino-dipeptidase 2.7 and aspartate-amino-transferase 2.1. Cathepsin A could only be found in the reticulocytes. Aspartate-amino-transferase (GOT) is admittedly not a proteolytic enzyme in the narrow sense of the term, but was determined as in the previous studies. It may possibly play an important role in the synthesis of glutathione [10].

Changes in the N-fractions are involved with these modifications in the enzyme profile. The total nitrogen content of the cells is insignificantly

reduced in the reticulocytes. The protein residue appears to be increased in the reticulocytes; however, due to the considerable scatter, the difference has likewise not been demonstrated. The erythrocytes are distinguished from the reticulocytes by an increase in hemoglobin at the expense of the stroma, residual-N and perhaps the protein residue as well. The residual-N and the amino-N in the reticulocytes are twice as high as in the erythrocytes. These results theoretically correspond to the *in vitro* findings of Schweiger, Rapoport and Schoelzel [11-14].

In two other animals, the same values (except for those enzymes which exhibited no clear changes in activity) was determined as a function of time (Figure 1).

The hematological modifications (upper part of the figure) correspond to the findings of other authors [15-17].

A rapid normalization of the hemoglobin concentration of the blood was achieved by the production of large cells which were highly pigmented. The number of cells as well as the other parameters (reticulocyte count, mean corpuscular volume (MCV), mean corpuscular hemoglobin content (MCH) approached an equilibrium state only at the end of the test period. According to Borsook et al. [18] the first, short-lived cell generation was derived directly from the polychromatic erythroblasts.

The peaks of enzyme activity (Figure 1 center) developed in a clear sequence. Five days after the start of the experiment, the proteinases had already reached their maximum. Both then dropped off sharply. Cathepsin A could no longer be detected after the eleventh day, while acid proteinase persisted, exhibiting a lower activity that lay within the normal range. The maxima of the exopeptidases followed those of the proteinases, with an interval of 1-2 days. Their initially rapid drop slowed after several days. At the end of the experimental period, the activities of glycyl-leucine, glycyl-glycine and imino-dipeptidase as well as aspartate-amino-transferase was still 3.2, 2.3, 1.8 and 1.5 times the initial values.

A clear sequence can also be seen in the behavior of the N-fractions. There is an interval of 2 days between the maxima or the stroma-N, residual-N

and amino-N and the maximum and minimum values of the protein residue, Hb and total-N. Residual-N and amino-N fall into the normal range beginning with the eleventh day, while the other N-fractions achieve this state only toward the end of the experiment. There is a definite correlation between the hemoglobin and total-N and an inverse relationship between these fractions and the protein residue.

Discussion

On the basis of the activity curves plotted during the maturation and aging of the erythrocytes, the proteases can be divided into 3 groups:

1. Cathepsin A can be found only in the acute phase of the reticulocytic crisis.

2. Of the exopeptidases, glycyl-leucine-dipeptidase, glycyl-glycin--dipeptidase and imido-dipeptidase initially increase rapidly during regeneration, then gradually decrease. GOT behaves analogously.

3. On the other hand, the other enzymes must exhibit a high degree of stability. An increase in leucine-amino-peptidase proved to be a sensitive criterion of increased regeneration in our studies of clinical material [5]. In the case of the rabbit, however, this appears to be a permanent condition to a certain extent, since in this case the lifetime of the erythrocytes is only 65-70 days [19]. However, this difference in cell metabolism does not appear to have any influence upon tripeptidase.

The total proteinase activity in the acid range exhibits behavior similar to that of the enzymes in group 2. Similar results have already been obtained *in vivo* by bleeding [20-22] and recently in erythrocyte maturation *in vitro* [23-25]. The differences between the young and old cells are so great that they can be successfully defended as age differentiation of erythrocytes [26].

"Acid proteinase", however, does not appear to be a homogeneous enzyme. It may contain at least two proteinases, of which one disappears with the substrate specificity of a cathepsin A during the reticulocytic crisis. The persistent activity which can be determined only in the mature erythrocytes is localized in the stroma and may correspond to the purified enzyme obtained by Morrison and Neurath [27]. Its pH optimum and the lack of an ability to be /211

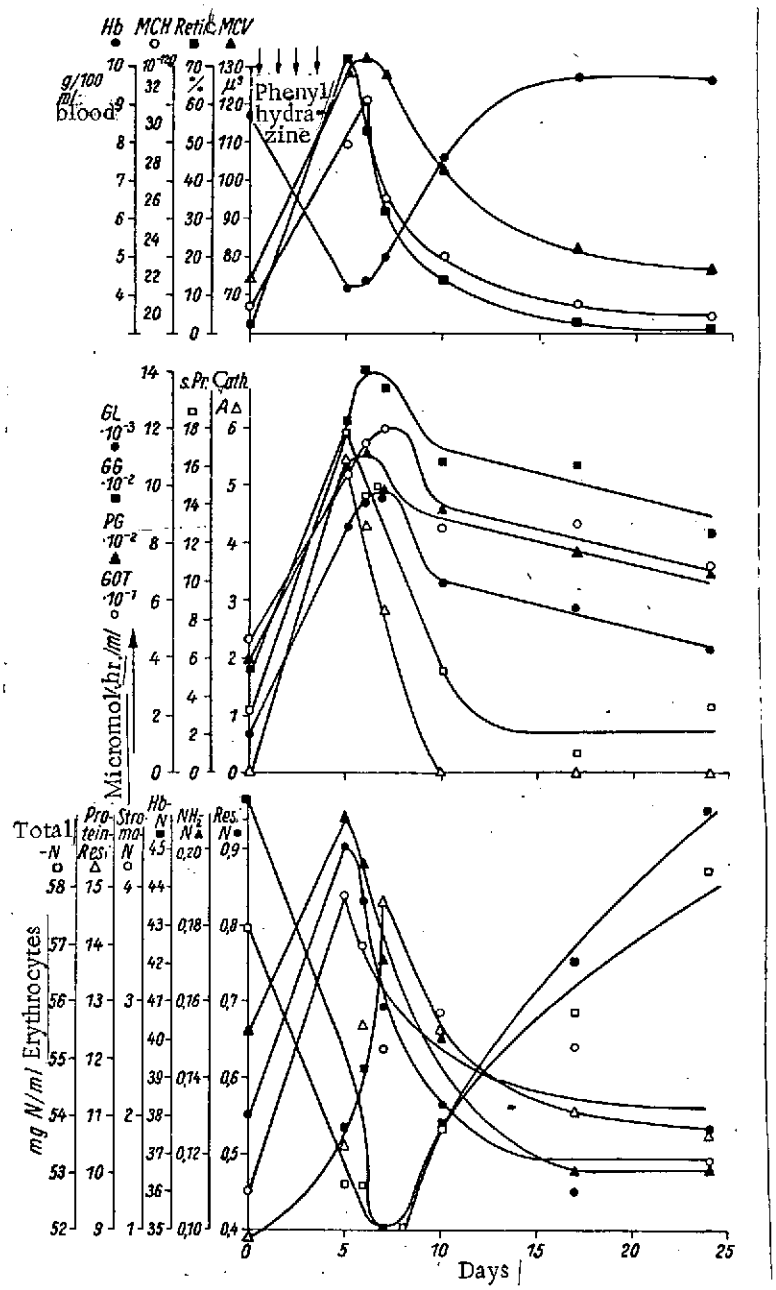


Figure 1. Erythrocytometric Findings (Top), Proteolytic Enzymes (Center), and Nitrogen Fractions (Bottom) During Erythrocyte Maturation. Each point constitutes an average of two animals. Treatment of animals and methods of determination are described under the methods. The follow- enzymes were determined: acid proteinase (s. Pr.), cathepsin A (Cath. A), glycyl-leucine-dipeptidase (GL), glycyl-glycin- -dipeptidase (GG), imino-dipeptidase (PG) and aspartate- amino-transferase (GOT). (Commas indicate decimals)

activated by L-cystein (10 mM), as indicated by our findings as well, correspond to the behavior of a cathepsin D [28]. Although the dimensions of both activities cannot be compared directly for methodological reasons, cathepsin A probably controls a significant part of the proteinase activity during the acute reticulocyte crisis.

A comparison of the enzymological and morphological findings provides important information about the causes of the different behavior of proteases in cell maturation. During the period in which cathepsin A can be detected in our experiments (Figure 1), the mean cell volume (MCV) is approximately twice normal. These cells are probably produced directly from the polychromatic erythroblasts, skipping the stage of orthochromatic erythroblasts, and have a short lifetime [18]. The volume curve (MCV) drops off more slowly as compared to the reticulocyte curve, i.e., these cells circulate for a short time as large erythrocytes after they have been dereticulated [16, 29]. The parallel course of the curves of cathepsin A and the reticulocytes shows that the cathepsin activity of these cells disappear simultaneously with ribonucleic acid. The curves of the exopeptidases on the other hand correspond to the volume curve and indicate by their shape that they are composed of one rapidly and one slowly decreasing component (later cell generations with normal lifetimes).

The average hemoglobin content of the individual erythrocytes (MCH) and the mean cell volume (MCV) become normal 19 days after the reticulocytic crisis. The cells of the first generation must therefore have disappeared by this point in time at the latest from the circulation. In this phase, the exopeptidases shown in Figure 1 still exceed the initial value. Normalization can only be anticipated when a normal age composition of the cell population is in effect.

The same problem was discussed by Rapoport et al. [30] using transfusion experiments. The study of the enzyme activities of transfused rabbit reticulocytes in non-anemic recipient animals revealed two groups of which one (respiration, succinate oxidase, pyrophosphatase and glucose-6-phosphate dehydrogenase) decreased relatively slowly while the other (glutaminase, see [31]) decreased rapidly. This rapid decline can only be explained by a maturation process which overlaps that of the elimination of the large, immature cells.

A further explanation for the different types of behavior of the enzymes is offered by their location. Cathepsin A may possibly be particle-bound. From the liver homogenate, it was sedimented out primarily with the mitochondrial fraction [32]. In the fractioning experiments with reticulocytes, proteinase was found primarily in the stroma, while it occurred in small amounts in the supernatent fluid and in the ribosomes [33]. Phenylhydrazine and oxidized glutathione inhibit proteinase in the reticulocyte hemolysate, but not in cell suspensions [34, 35]. This is also an indication that the enzyme in the /212
in the intact cells occurs in the bound form. In our own experiments [36] we found that repeated freezing and thawing the proteinase-activity of reticulocytes can be released much more rapidly than from erythrocytes. On the other hand, the exopeptidases are dissolved primarily in the cytoplasm ([37, 49] and likewise LAP).

The function of the proteolytic enzymes, up to a certain degree, can consist of changes in the N-fractions. The reduced total nitrogen content of the reticulocytes increases during cell maturation. This exogenic nitrogen, however, accounts for only 30-60% of the N-requirement for hemoglobin synthesis. The missing component must be made up from the catalysis of cell protein. The source is the stroma, by virtue of its products of catabolism (peptides, amino acids) in the residual-N-fraction [11-14]. On the other hand, the protein residue probably contains an intermediate stage of hemoglobin synthesis, since its increase corresponds to a decrease in the stroma-N and residual-N and its decrease corresponds to an increase in hemoglobin. (Figure 1, bottom). Such heme-free hemoglobin precursors were isolated by Greenough et al. [38]. A proteolytic breakdown of this protein fraction is therefore unlikely, because the proteinase activity has already been markedly reduced by its decomposition. The remaining part of the protein residue might correspond to the enzymes which are not bound to the stroma. Even in mature human erythrocytes, in addition to hemoglobin, two protein fractions have been determined electrophoretically which together make up 4% of the total protein in the hemolysate [39].

These findings lead to the following diagram of protein metabolism during erythrocyte maturation:

$$\text{Stroma} \xrightarrow{\text{Cathepsin A}} \text{Peptide} \xrightarrow{\text{Exopeptidases}} \text{Amino Acid Pool} \rightarrow \text{Hb-Precursors} \rightarrow \text{Hb}$$

Exogenic Amino Acids

The liberation of nitrogen from the stroma during incubation of reticulocytes was markedly inhibited by 2,4-dinitrophenol [11-14]. From this we can conclude that the effectiveness of the responsible enzyme, cathepsin A, is related to certain energy preconditions such as has been found to be the case for other proteinases [40-43], [48] and the breakdown of ribonucleic acid in the reticulocytes [44].

In intracellular proteolysis, proteinases probably play a key function. This follows (1) from their function as endopeptidases, (2) from their limiting activities – they are 1-3 orders of magnitude lower than those of the exopeptidases – and (3) from the sequence of the peaks (Figure 1, center). The protein catabolism, however, is limited not only quantitatively but also chronologically. Following the loss of cathepsin A, there may only be minimum decomposition of the stroma and aged enzymes *in vivo*. *In vitro*, in the case of incubation of erythrocytes with the exception of ammonia [31, 45], only small amounts of /213
substances containing N are liberated [11, 12, 14]. It is only after hemolysis that a definite autolysis can be seen at pH 3.5. This creates the impression of a discontinuous proteolysis, which reaches its maxima during maturation and final catabolism of the cells and exhibits its minimum during the maturation of the cells.

The optimum effective conditions of the exopeptidases are sufficiently broad to guarantee nearly complete effectiveness in the physiological pH range of the cytoplasm. In the subcellular particles and in the stroma, there may be other conditions. Furthermore, the pH optima of the cathepsins are substrate-dependent. Thus, cathepsin D hydrolyzes hemoglobin optimally at pH 3.0, but albumin at pH 4.2 [28]. It is likely that a pH decrease would cause the final decomposition of the erythrocytes through the activation of stroma proteinase and denaturation of the cell proteins.

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Translated for the National Aeronautics and Space Administration under contract No. NASw-2485 by Techtran Corporation, P.O. Box 729, Glen Burnie, Maryland, 21061. Translator: W. J. Grimes, M.I.L.